

Interleukin-15 is not required for the induction or maintenance of orally induced peripheral tolerance

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SUMMARY

Orally induced tolerance is a physiologically relevant form of peripheral tolerance, which is believed to be important for the prevention of pathological immune responses in the gut. Of several mechanisms proposed to mediate oral tolerance, one that has received much attention recently is the concept of regulatory CD4⁺ T cells. As recent studies have suggested that interleukin (IL)-15 may be important for the differentiation and maintenance of regulatory CD4⁺ T cells, we have examined the role of IL-15 in oral tolerance, using a soluble form of the IL-15 receptor (sIL-15R) which blocks the biological effects of IL-15 *in vivo*. Oral tolerance induced by feeding mice ovalbumin (OVA) in a low-dose regimen believed to induce regulatory T cell activity was not affected by the administration of sIL-15R during either the induction or maintenance phase of tolerance. Thus, oral tolerance does not involve an IL-15-dependent mechanism.

Keywords cell survival, IL-15, oral tolerance, regulatory T cell

INTRODUCTION

The ability of the intestinal immune system to prevent overwhelming inflammation in the gut reflects the fact that food antigens and commensal bacteria induce a state of systemic hyporesponsiveness known as oral tolerance (reviewed in Garside and Mowat¹). Several mechanisms have been described to be important in oral tolerance, with high doses of fed antigen appearing to cause clonal deletion and/or anergy of antigen-specific cells, whereas repeated low doses of antigen are proposed to induce active suppressor mechanisms mediated by regulatory T cells (T_{reg}).²

T cells with regulatory capacity have been recognized in oral tolerance for many years and although described originally to be CD8⁺ suppressor T cells,³ most work now indicates that, as in other models of peripheral tolerance, a subset of CD4⁺ T_{reg} is likely to be important.^{4–8} A number of types of CD4⁺ T_{reg} have been described in different

models of tolerance, including T_H3⁹ T_R1¹⁰ and CD4⁺CD25⁺^{11,12} cells which may act through soluble factors such as interleukin (IL)-10^{10,13,14} or transforming growth factor (TGF)- β ^{15,16} or via cell-contact mechanisms such as CTLA-4^{17,18} or surface-bound TGF- β .¹⁹ To date, very little is known of the factors that determine the differentiation and activity of T_{reg} *in vivo* or in mucosal immunity specifically. Of particular note, the relationship between the different subsets and their functions is not clear, with *in vitro* generated T_{reg} not having been fully characterized *in vivo* and vice versa. As it can be difficult to examine T_{reg} in the *in vivo* situation, here we have attempted to define the role of one such *in vitro* defined subset, the T_R1 cell, in oral tolerance, by exploiting their dependence on IL-15 as an essential growth factor.

Described originally as a T cell growth factor,²⁰ IL-15 also plays an important proinflammatory role in disease, being released by macrophages and tissue cells early in an immune response.²¹ In this context, several effects on T cells have been described, the most significant being the generation and maintenance of memory T cells.^{22,23} However, more recently, it has been shown that in humans IL-15 may also be important for the expansion of certain T_{reg} *in vitro*.^{24–26} Specifically, IL-15 has been shown to induce and support the proliferation of IL-10-producing human T_R1 cells generated *in vitro* by repeated stimulation

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with IL-10²⁴ as well as human T_{reg} generated *in vitro* by costimulation blockade.²⁶ Resolving this apparent conflict between the pro- and anti-inflammatory effects of IL-15 may have important consequences for its therapeutic manipulation.

Although T_R1-like cells have been shown to cause bystander suppression of IBD after feeding the appropriate antigen in a murine, *in vivo* model¹⁰ their exact role has not been established in the intact immune system or in oral tolerance and it is not clear if IL-15 has any role in these processes in mice. Here we took advantage of the availability of a high-affinity soluble form of the IL-15R α which blocks activity *in vivo* to examine whether IL-15 is important for the maintenance of oral tolerance in a model which is described to induce regulatory mechanisms.

METHODS

Mice

Female BALB/c mice (Harlan Olac, Bicester, UK) were kept under specific pathogen-free conditions at the Central Research Facility, University of Glasgow and used between 6 and 8 weeks of age in line with local and Home Office guidelines.

Preparation of soluble IL-15R α

Soluble IL-15R α (T1) and the non-binding mutant protein M4²⁷ were prepared as described previously.²⁸ T1 spans the entire extracellular domain of the murine IL-15R α chain, whereas M4 contains a single site-directed mutation in which the third cysteine of the 'Sushi domain' of the α -chain has been replaced with an aspartic acid. This mutant has no ability to bind IL-15.²⁷ Recombinant histidine-tagged proteins were expressed in *Escherichia coli* (XL-1 Blue; Stratagene, Amsterdam, the Netherlands) by isopropyl- β -D-thiogalactoside (Stratagene) induction and purified by a nickel-agarose purification system (Qiagen, Crawley, West Sussex, UK) according to the manufacturers' recommendations. Purified proteins were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The purity was > 97% for all recombinant proteins and lipopolysaccharide (LPS) was undetectable (< 0.01 ng/mg) by the *Limulus* amoebocyte test (E-toxate; Sigma-Aldrich, Poole, Dorset, UK).

Carrageenan-induced footpad inflammation

Local inflammation was induced by subcutaneous (s.c.) injection of 300 μ g of λ -carrageenan (Sigma-Aldrich) dissolved in 50 μ l phosphate buffered saline (PBS) into the left hind footpad. Footpad swelling was assessed by caliper measurement (Kroeplin, Munich, Germany) 4–48 hr after injection and the degree of inflammation was determined by calculating the difference between injected and uninjected contralateral control footpads. To suppress inflammation, 5 μ g of the T1 or M4 forms of the sIL-15R α was mixed together with the subcutaneous injection of carrageenan. Alternatively, mice were treated with 40 μ g T1 given intraperitoneally (i.p.) at the time of carrageenan injection.

Induction and assessment of oral tolerance

Oral tolerance was induced by feeding 1 mg of ovalbumin (OVA; Sigma-Aldrich) dissolved in 0.2 ml PBS by oral gavage using a stainless steel gavage needle for 5 consecutive days. Control mice were fed PBS alone. Mice received 40 μ g of T1 or M4 dissolved in PBS by daily i.p. injection starting 2 days after the first feed and continuing until 15 days after the last. Seven days after the last feed, mice were immunized with 100 μ g OVA emulsified in 50 μ l complete Freund's adjuvant (CFA; Sigma-Aldrich) in the hind footpad. Twelve days after immunization, draining lymph nodes were harvested and the cells assessed for OVA-specific proliferation and cytokine production, while systemic DTH and antibody responses were assessed 20 days after challenge as described previously.²⁹

OVA-specific proliferation *in vitro*

Single cell suspensions of draining popliteal lymph nodes were prepared by rubbing through Nitex mesh (Cadisch, London, UK). After washing, cells were counted and resuspended at 10⁶ cells/ml in 200 μ l RPMI-1640 containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine and 50 μ g/ml fungizone (all Life Technologies, Paisley, UK) either alone or with 1 mg/ml OVA. The cells were then cultured for 120 hr in flat-bottomed 96-well tissue culture plates (Costar, Nucleopore, High Wycombe, UK) at 37° in a 5% CO₂ humidified incubator. Proliferation was assessed by addition of 1 μ Ci/well [³H]-TdR for the last 18 hr of culture. DNA-bound radioactivity was harvested onto glass fibre filter mats and thymidine-incorporation measured on a 1205 Betaplate scintillation counter (both Wallac Oy, Turku, Finland).

Measurement of OVA-specific cytokine production

Lymph node cells were resuspended at a final concentration of 4 $\times$ 10⁶ cells/ml and 1 ml aliquots cultured in 24-well plates (Costar) for 48 hr either in medium alone or with 1 mg/ml OVA. Supernatants were centrifuged at 13 000 g and stored at –20° until cytokine levels were quantified by sandwich enzyme-linked immunosorbent assay (ELISA) protocol, using appropriate specific antibodies [interferon (IFN)- γ capture clone RF-6A2, detection clone XMG1.2; IL-5 capture clone TRFK5, detection TRFK4; all Pharmingen, Oxford, UK]. Recombinant cytokines (R&D Systems, Abingdon, Oxon, UK) were used as standards and the levels in supernatants calculated by using a Dynatech MR5000 automatic microplate reader and Revelation software (both Dynex Technologies, West Sussex, UK).

In vivo assessment of oral tolerance

Serum samples were collected and analysed for levels of OVA-specific IgG. Briefly, venous blood was collected into heparinized capillary tubes (Hawksley & Sons Ltd, Lancing, Sussex, UK), the serum separated by centrifugation at 450 g for 10 min and stored at –20° until use. Immulon-4 ELISA plates (Dynatech, Billingshurst, West Sussex, UK) were coated overnight at 4° with 10 μ g/ml OVA in 0.05 M carbonate buffer (pH 9.3) and non-specific binding sites

blocked for 2 hr using phosphate buffered saline (PBS)/0.05% Tween 20 (Sigma-Aldrich) supplemented with 10% FCS. Samples were then added diluted 1 : 400 in PBS/Tween 20 with 10% FCS for 2 h at 37°. After incubation, plates were washed and incubated with 100 µl/well peroxidase-conjugated anti-mouse IgG (Sigma-Aldrich) before visualization using 3,3',5,5'-tetramethylbenzidine peroxidase substrate (Insight Biotechnologies, Wembley, Middlesex, UK). Plates were read at 630 nm using a microplate reader.

OVA-specific delayed-type hypersensitivity (DTH) responses were assessed as described previously.³⁰ Briefly, mice were anaesthetized using halothane, the thickness of the unimmunized rear footpad measured using calipers and 100 µg heat-aggregated OVA in 50 µl saline was injected subcutaneously. The increases in individual footpad thickness were measured 24 hr later. OVA-specific delayed-type hypersensitivity (DTH) responses were calculated by subtracting the increment found upon challenge of naive BALB/c mice.

Statistical analysis

Unless stated otherwise, results are represented either as the mean $\pm$ 1 SD or as readings from individual animals, as appropriate. Data with normal distribution were analysed using Student's *t*-test while data sets with skewed distribution were evaluated using a Mann-Whitney *U*-test. Statistical analysis was performed using Minitab software (State College, PA).

RESULTS

Soluble IL-15R α reduces carrageenan-induced inflammation

We used the acute inflammatory response induced by carrageenan to confirm the *in vivo* biological activity of sIL-15R α . BALB/c mice were treated with 40 µg sIL-15R α i.p. or 5 µg s.c. injected with 300 µg of λ -carrageenan and the subsequent inflammatory response measured. Footpad swelling was evident as early as 4 hr after injection of carrageenan and this inflammation persisted for at least 48 hr

(Fig. 1). The non-binding control receptor M4 had no significant effect on local inflammation (Fig. 1a). However, as described previously,²⁷ treatment with T1 reduced carrageenan-induced inflammation significantly when administered s.c. along with carrageenan (Fig. 1a) or i.p. at the time of challenge (Fig. 1b), demonstrating inhibition of IL-15 *in vivo*. Importantly, a single systemic dose of 40 µg T1 administered i.p. was also able to block IL-15 function, reducing carrageenan-induced inflammation in the footpad for at least 24 hr.

Blockade of IL-15 does not prevent oral tolerance

As IL-15 has been reported to be important for the maintenance of a subset of human regulatory T cells and low-dose oral tolerance has been described to induce cells with suppressive activity, we examined the effects of IL-15 blockade on this form of tolerance. Mice were fed PBS or 1 mg OVA for 5 consecutive days and treated daily with 40 µg T1 or M4 i.p. from the day of the third feed until 15 days following the last feed. Mice were challenged with OVA/CFA, and 12 days following immunization antigen-specific proliferative responses by draining lymph node cells were assessed *in vitro*. Mice fed PBS and treated with control M4 protein were primed efficiently following immunization with OVA in adjuvant, as determined by specific proliferative responses (Fig. 2a) or the production of Th1 and Th2 cytokines *in vitro* (Fig. 2b,c). The responses were not affected by neutralization of IL-15 function *in vivo*. In mice treated with the inactive form of the sIL-15R α (M4), oral administration of OVA before immunization-induced systemic tolerance, as shown by significantly reduced proliferative responses and decreased production of IL-5 and IFN- γ , when lymph node cells were restimulated with OVA *in vitro*. Similar results were obtained in OVA-fed mice treated with neutralizing sIL-15R α (T1), with identical suppression of proliferative responses, as well as of IL-5 and IFN- γ production after challenge with OVA/CFA (Fig. 2), suggesting that oral tolerance was induced efficiently in the absence of IL-15.

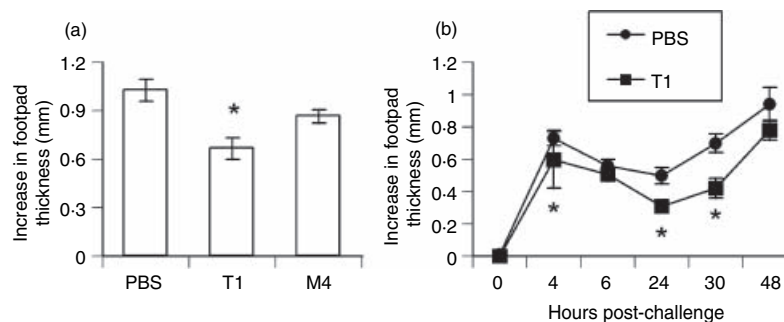


Figure 1. Effects of neutralizing IL-15 on carrageenan-induced local inflammation. (a) Mice were injected with 300 µg λ -carrageenan in PBS and treated with 5 µg sIL-15R α (T1) or inactive sIL-15R α (M4) mixed with the carrageenan (a), or 40 µg T1 i.p. at the time of carrageenan (b), and footpad thickness measured 4 h (a) or 4–48 h later (b). The results shown are the mean increase in footpad thickness $\pm$ standard error of mean for five mice per group (**P* < 0.05 versus PBS-treated control).

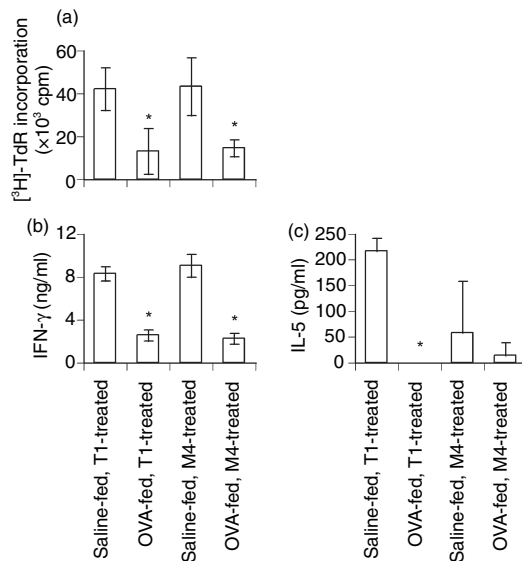


Figure 2. Effects of neutralizing IL-15 function on oral tolerance *in vitro*. OVA-specific proliferative responses were measured 12 days after subcutaneous challenge of OVA- or PBS-fed mice with OVA/CFA. Mice were treated daily i.p. with T1 or M4 from 2 days after the first feed and for 17 days thereafter. The results shown are the mean [³H]-TdR incorporation in c.p.m. (± 1 SD) for triplicate cultures of cells from three mice per group (a) or cytokine production by pooled cells from each group as assessed by ELISA (shown as concentration ± 1 SD) (b and c) (* $P < 0.05$ versus saline-fed, same treatment).

Blockade of IL-15 does not prevent the *in vivo* consequences of oral tolerance

To assess the effects of oral tolerance on systemic immunity *in vivo*, serum OVA-specific IgG and systemic DTH responses were measured 20 days after challenge with OVA/CFA. As shown in Fig. 3, DTH responses were induced in saline-fed mice treated with the inactive M4 receptor upon rechallenge with heat-aggregated OVA, and this inflammation was significantly suppressed in mice previously fed OVA. Identical suppression of DTH responses was found in OVA fed mice treated with neutralizing sIL-15R α (Fig. 3). OVA-specific serum IgG antibody responses were also reduced significantly in T1 treated orally tolerized mice compared with saline controls (Fig. 4). The somewhat decreased IgG response found in M4-treated saline-fed mice was also decreased further by feeding OVA, although this was not statistically significant.

Together these results indicate that IL-15 signalling is not required for the long-term maintenance of oral tolerance.

DISCUSSION

Many groups have described an important role for a population of regulatory or suppressor cells following the induction of oral tolerance,^{1,31,32} particularly when animals have been fed repeated low doses of antigen,² but the

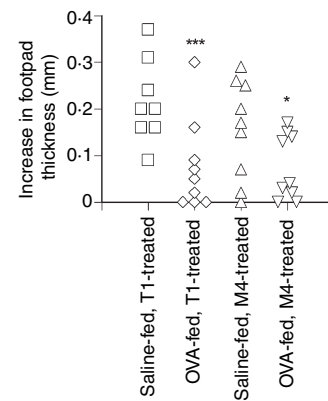


Figure 3. Effect of sIL-15R α treatment on oral tolerance *in vivo*. Mice were tolerized and treated as described in Figure 2 and systemic DTH responses were assessed 20 days after immunization with OVA/CFA by injecting 100 μ g heat-aggregated OVA. The results shown are the increases in footpad thickness from individual mice (* $P < 0.05$, *** $P < 0.005$ versus saline-fed, same treatment).

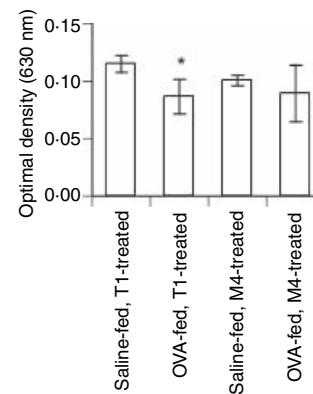


Figure 4. Effect of IL-15 inhibition on oral tolerance *in vivo*. Serum IgG were assessed 20 days after immunization with OVA/CFA by ELISA. The results are shown as the mean OD of five mice per groups (± 1 SD) (* $P < 0.05$ saline-fed control).

factors responsible for promoting or maintaining these T_{reg} are unknown. One of the best-studied populations of T_{reg} *in vitro* are the IL-10-producing T_R1 cells, whose differentiation and survival, at least in human studies, is favoured by the presence of IL-10 and IL-15, respectively.²⁴ As these T_R1-like cells have been shown to have regulatory activities in the intestine of mice,¹⁰ the microenvironment of the gut is high in IL-10 and intestinal epithelial cells produce IL-15,³³ we hypothesized that oral tolerance might require the presence of IL-15 *in vivo*. To examine this possibility we administered a soluble form of the IL-15R α , which has been described previously to antagonize IL-15 function in *in vivo* models of arthritis, allograft rejection and parasite infection.^{27,28,34,35} Inhibition of IL-15 function at the time of, and after, feeding antigen had no effect on the induction or expression of systemic tolerance, as assessed *in vitro* or *in vivo*. This suggests that IL-15 is not important for the

persistence of this form of oral tolerance. There are a number of possible reasons for the results observed. First, it is possible that the inhibition of IL-15 function *in vivo* is not complete, or treatment with the sIL-15R α does not antagonize IL-15 function at sites that may be important for the induction of T_{reg}. Expansion of T_{reg} *in vitro* by IL-15 required high doses of cytokine²⁶ and thus treatment with sIL-15R α in these experiments is likely to reduce the availability of IL-15 *in vivo* below these concentrations required. The biological activity of the sIL-15R treatment regimen was confirmed by the fact that intraperitoneal administration of the bioactive T1 protein abrogated IL-15 function in carrageenan-induced inflammatory responses in the footpad. Nevertheless, it remains possible that IL-15 function is not inhibited in mucosal tissues by i.p. delivery of sIL-15R. We therefore examined the systemic induction of oral tolerance by assessing tolerance to subcutaneous challenge with antigen following feeding.

Although the administration of multiple low doses of OVA as used here is able to induce active suppression,^{2,36} it is possible that any regulatory T cells induced in our system are not dependent on IL-15 function *in vivo*. Of the different subsets of T_{reg} described in oral tolerance, none are exactly equivalent to the *in vitro*-derived IL-15-dependent T_R1 cells and the role of IL-15 in the maintenance of *in vivo*-generated Th3 or CD4⁺CD25⁺T_{reg} (each of which have been described in oral tolerance^{7,8,37}) has not been examined. Furthermore, it has not yet been established formally that IL-15 plays a role in the generation or maintenance of murine T_{reg}. Thus, different populations of T_{reg} in different species may have differing requirements for their long-term survival and/or expansion.

In summary, the results shown here demonstrate that IL-15 function is not important for the maintenance of oral tolerance. The fact that IL-15 antagonism had no effect on tolerance induction suggests that either, contrary to previous reports, T_{reg} are not induced in this model of oral tolerance or that IL-15 is not required for maintenance of these cells in mice. These findings also have important implications for the therapeutic use of IL-15 antagonism, suggesting that these procedures are not likely to cause breakdown of established tolerance.

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